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Construction of *Aeromonas salmonicida* subsp. *achromogenes* AsaP1-toxoid strains and study of their ability to induce immunity in Arctic char, *Salvelinus alpinus* L.

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Abstract

The metalloendopeptidase AsaP1 is one of the major extracellular virulence factors of *A. salmonicida* subsp. *achromogenes*, expressed as a 37-kDa pre-pro-peptide and processed to a 19-kDa active peptide. The aim of this study was to construct mutant strains secreting an AsaP1-toxoid instead of AsaP1-wt, to study virulence of these strains and to test the potency of the AsaP1-toxoid bacterin and the recombinant AsaP1-toxoids to induce protective immunity in Arctic char. Two *A. salmonicida* mutants were constructed that secrete either $AsaP1_{E294A}$ or $AsaP1_{Y309F}$. The secreted $AsaP1_{Y309F}$-toxoid had weak caseinolytic activity and was processed to the 19-kDa peptide, whereas the $AsaP1_{E294A}$-toxoid was found as a 37-kDa pre-pro-peptide suggesting that AsaP1 is auto-catalytically processed. The LD_{50} of the $AsaP1_{Y309F}$-toxoid mutant in Arctic char was significantly higher than that of the corresponding wt strain, and LD_{50} of the $AsaP1_{E294A}$-toxoid mutant was comparable with that of an AsaP1-deficient strain. Bacterin based on $AsaP1_{Y309F}$-toxoid mutant provided significant protection, comparable with that induced by a commercial polyvalent furunculosis vaccine. Detoxification of AsaP1 is very hard, expensive and time consuming. Therefore, an AsaP1-toxoid-secreting mutant is more suitable than the respective wt strain for production of fish bacterins aimed to protect against atypical furunculosis.

Keywords: Aeromonas salmonicida subsp. *achromogenes*, Arctic char, AsaP1-toxoid, bacterin, extracellular protease AsaP1, *Salvelinus alpinus* L.

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Introduction

Aeromonas salmonicida subsp. *achromogenes* is a pathogen of salmonids and many other fish species, causing atypical furunculosis, which can cause high economic loses (Gudmundsdottir & Bjornsdottir 2007).

Commercially available vaccines against furunculosis are bacterins that are most often polyvalent and contain killed *A. salmonicida* subsp. *salmonicida* as an antigen. Bacterins based on *A. salmonicida* subsp. *salmonicida* can cross-protect against *A. salmonicida* subsp. *achromogenes*, but all available publications are based on experimental

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vaccinations with monovalent bacterinssub (Gudmundsdottir & Bjornsdottir 2007). However, there have been problems on many fish farms in Iceland with atypical furunculosis occurring in Arctic char, *Salvelinus alpinus* L., vaccinated with commercial furunculosis vaccines, especially in stressful conditions and after approximately 1600 degree days in culture (Gísli Jónsson, Veterinary Officer for Fish Diseases in Iceland, personal communications). Studies show that efficacy of inactivated extracellular products (ECP) to evoke protective immunity in fish against bacterial infections and the protection induced by the ECP of *A. salmonicida* subsp. *achromogenes* correlate with an increased antibody response against the extracellular protease AsaP1 (Santos *et al.* 1991; Magariños *et al.* 1994; Gudmundsdottir & Magnadottir 1997; Gudmundsdottir *et al.* 1997; Collado *et al.* 2000).

AsaP1 is a metalloendopeptidase that is expressed by *A. salmonicida* subsp. *achromogenes* as a 37-kDa pre-pro-peptide, which is processed to the highly toxic 19-kDa mature enzyme (Gudmundsdottir, Hastings & Ellis 1990; Arnadottir *et al.* 2009). Previously, four different AsaP1-toxoids, AsaP1$_{E294A}$, AsaP1$_{E294Q}$ AsaP1$_{Y309F}$ and AsaP1$_{Y309A}$, were constructed and recombinantly produced in *E. coli* (Schwenteit *et al.* 2013). Toxoid AsaP1$_{Y309F}$, expressed in *E. coli*, showed reduced caseinolytic activity and was processed to a 22-kDa peptide similar in size to the recombinant wild type (wt) AsaP1, whereas the other toxoids showed no caseinolytic activity and remained as the unprocessed 37-kDa pre-pro-peptide. All four AsaP1 mutants induced an antibody response in Arctic char. Before its use in fish vaccines, the AsaP1 wt must be detoxified, but the necessary procedure is harsh, expensive and time consuming, as it involves overnight dialysing in PBS containing protease inhibitors and incubation in formalin for 7 days at 22 °C, followed by dialysis in water (Gudmundsdottir *et al.* 1997). There is no available data showing that the prolonged procedure harm important bacterial antigens, but treatment of antigens that induce protection should always be kept at minimum.

The main aim of this study was to construct an *A. salmonicida* subsp. *achromogenes* strain that produces an AsaP1-toxoid instead of the wt enzyme that can be used for production of fish bacterins.

Materials and methods

Bacterial strains, plasmids and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 1. *Aeromonas* strains were grown in brain heart infusion media (BHI, Fluka/Sigma-Aldrich) and *E. coli* strains in Luria–Bertani-media (LB, Roth). Growth medium was supplemented with 1.5% (w/v) agar (Difco) when appropriate. Liquid cultures were routinely grown with agitation (180 rpm) at 16 °C for *Aeromonas* strains and 37 °C for *E. coli* strains. Bacteria were grown at room temperature (RT) in case of mating. All bacterial strains were stored at −80 °C.

Growth kinetics were analysed over 125 h in three parallel broth cultures, all inoculated to an initial cell density of 10^3–10^4 CFU mL^{-1} from precultures. Bacterial concentrations at T0 were determined by plating and counting colony forming units (CFU mL^{-1}) due to the low dilution. All later time points were analysed using FACS (Cyflow space, Partec).

Construction of *A. salmonicida* subsp. *achromogenes* AsaP1-toxoid mutants

Two *A. salmonicida* subsp. *achromogenes* AsaP1-toxoid mutants, secreting the toxoids AsaP1$_{E294A}$ and AsaP1$_{Y309F}$, respectively, were constructed by allelic exchange. All primers used for isolation of the *asaP1* gene region, mutant construction and sequencing are listed in Table 2. The toxoid genes, previously constructed in pJoe (Schwenteit *et al.* 2013), were cloned into the suicide vector pDM4 using *Sac*I and *Spe*I restriction sites (Milton *et al.* 1996). The resulting plasmids, pDM4_*asaP1::E294A* and pDM4_*asaP1::Y309F*, were first transformed into *E. coli* DH5α*pir* (Dunn, Martin & Stabb 2005), due to better transformation rate, further isolated and transformed into *E. coli* S17.1 (Simon, Priefer & Pühler 1983), which is able to conjugate to *A. salmonicida* subsp. *achromogenes*. The *pir* gene is essential to replicate the suicide vector pDM4 (Kolter, Inuzuka & Helinski 1978). The two bacteria were mixed in a ratio of 10:1 *A. salmonicida* subsp. *achromogenes* wt strain Keldur265-87: *E. coli* S17.1, spotted on BHI agar and incubated at RT for 18 h. Bacteria was first selected on BHI plates containing 50 μg mL^{-1} Amp and 25 μg mL^{-1} Cam for a single crossover and further a double crossover using 5% sucrose BHI agar, as

Table 1 Bacterial strains and plasmids used in the study

Strains or plasmids	Properties	Source
Strains		
A. salmonicida subsp. *achromogenes*		
Keldur265-87	Isolate from diseased Atlantic salmon, *Salmo salar* L.	Gudmundsdottir *et al.* (1990)
Keldur265-87-2	Δ*asaP1*::kan derived from Keldur265-87	Arnadottir *et al.* (2009)
Keldur265-87-4	Δ*asaP1*::$asaP1_{E294A}$ derived from Keldur265-87	This study
Keldur265-87-5	Δ*asaP1*::$asaP1_{wt}$ derived from Keldur265-87-4, using pDM4_*asaP1wt*	This study
Keldur265-87-6	Δ*asaP1*::$asaP1_{Y309F}$ derived from Keldur265-87	This study
Keldur265-87-7	Δ*asaP1*::$asaP1_{wt}$ derived from Keldur265-87-6, using pDM4_*asaP1wt*	This study
E. coli		
DH5α*pir*	supE44DlacU169 (Φ80lacZ_M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 *pir*	Dunn *et al.* (2005)
S17.1	thi pro hsdR hsdM+recA[RP4 2-Tc:: Mu-Km::Tn7(Tp^{r}Smr)Tra+] Mobilizing donor for conjugation	Simon *et al.* (1983)
Plasmids		
pJoe_*asaP1*::E294A	Ap^r, rha_{BAD}, vector for cloning and expression containing the allele of $AsaP1_{E294A}$ toxoid	Schwenteit *et al.* (2013)
pJoe_*asaP1*:: Y309F	Ap^r, rha_{BAD}, vector for cloning and expression containing the allele of $AsaP1_{Y309F}$ toxoid	Schwenteit *et al.* (2013)
pDM4	Cm^r; suicide vector with an R6K origin (*pir* requiring) and *sacBR* of *Bacillus subtilis*	Milton *et al.* (1996)
pDM4_*asaP1*::E294A	Cm^r; pDM4 containing the mutant allele of $AsaP1_{E294A}$ toxoid	This study
pDM4_*asaP1*::Y309F	Cm^r; pDM4 containing the mutant allele of $AsaP1_{Y309F}$ toxoid	This study
pDM4_*asaP1*wt	Cm^r; pDM4 containing the wt allele of AsaP1	This study

Table 2 Primers applied for isolation of genomic *asaP1* region, mutant construction and sequencing

Primer	Sequence	Application
*asaP1-Spe*I-f	5′-ACTAGTGGCGAGCACATAGTGCAGGGTGAGC-3′	Cloning
*asaP1-Sac*I-r	5′-GAGCTCCTATCTGGCAGTCGCGGCCC-3′	Cloning
asaP1-primer 1-r	5′-GTTGCCCTGGCTCAGCAGC-3′	Sequencing
asaP1-primer 2-f	5′-CTGCTCTAGGGTGAGTGGGC-3′	Sequencing
asaP1-primer 3-f	5′-CGGGTCTCTATGACATGAGTGC-3′	Sequencing
asaP1-primer 4-f	5′-CAAGCCGCTCACCTTTGAC-3′	Sequencing
asaP1-primer 5-f	5′-CTGTCGGTCAAGGCACACAG-3′	Sequencing
asaP1-primer 6-f	5′-GCCATCAGTTTCACCCGGTT-3′	Sequencing
asaP1-reg-f	5′-CGAGGTGAACGCCAAGCTG-3′	Isolation of *asaP1* region
asaP1-reg-r	5′-GGAACAGGCAAGCAGACCC-3′	Isolation of *asaP1* region

sucrose is lethal for cells expressing the levansucrase encoded on the suicide vector pDM4 (Gay *et al.* 1983). Colonies were patched on milk (1% skimmed milk) and Cam BHI plates (25 μg mL^{-1}). A clone was considered positive, if it was caseinase negative and Cam sensitive. The selected clones were single colony isolated and confirmed by sequencing the DNA locus encoding the *asaP1* gene.

Complementation of the mutations by reverse allelic exchange was performed according to the same protocol, but using pDM4 carrying the *asaP1* wt allele (pDM4_*asaP1wt*). The resulting transconjugates were screened for caseinase positive clones before confirmation by sequencing.

AsaP1-toxoid secretion and caseinolytic activity of the *A. salmonicida* subsp. *achromogenes* toxoid mutants

Expression and secretion of AsaP1 and its toxoids by *A. salmonicida* subsp. *achromogenes* were detected by Western blot analyses of the ECP using polyclonal murine α-AsaP1 antibodies, as

previously described (Gudmundsdottir *et al.* 2003). Caseinolytic activity was analysed spectrophotometrically at 450 nm (A_{450}) using azo-casein as substrate, as previously described (Gudmundsdottir 1996) and in a casein zymogram performed as described by Gudmundsdottir (1996), using 12% gels supplemented with 0.1% casein.

Experimental fish

Arctic char from Íslandsbleikja, Iceland, free of previous infections, according to standard routine diagnostic procedures performed at the Icelandic Fish-Disease Reference Laboratory, were used for vaccination and challenge. Fish were acclimatized for 1 week prior to treatment and anaesthetized with tricaine methane sulfonate (50 mg L^{-1}) and MS222 (PHARMAQ; Vistor hf.). Fish were marked with Visible Implant Fluorescent Elastomer dye (Northwest Marine Technology). Oxygen concentration, temperature and mortality were monitored daily over the experimental period, and the fish were fed to appetite with commercial pellets (Laxá hf.). The experiments were approved and performed according to the Icelandic Animal Research Authority (approval no. YDL03080041/023BE).

Estimation of LD_{50} and MDD of the *A. salmonicida* subsp. *achromogenes* AsaP1-toxoid mutants in Arctic char

Virulence of the *A. salmonicida* subsp. *achromogenes* AsaP1-toxoid mutants $AsaP1_{E294A}$ (Keldur265-87-4) and *A. salmonicida* subsp. *achromogenes* $AsaP1_{Y309F}$ (Keldur265-87-6) and their corresponding complemented strains (Keldur265-87-5 and Keldur265-87-7, respectively) were analysed by comparing the fifty per cent lethal dose (LD_{50}) calculated according to the method of Reed and Muench (Reed & Muench 1938) and by the mean day to death (MDD) calculated as previously published (Schwenteit *et al.* 2011). The wt strain Keldur265-87 and the AsaP1-deficient strain Keldur265-87-2 served as controls.

Arctic char (A: 44.7 ± 8 g and B: 31.1 ± 7.0 g), kept in 70-L tanks supplied with continuously running fresh water (A: 9 ± 1 °C and B: 11 ± 1 °C), were i.p. challenged with 100 μL of different concentrations of three times passed *A. salmonicida* subsp. *achromogenes* strains (Table 3). Infection was confirmed by re-isolation of the respective bacterium. The isolates were confirmed to be *Aeromonas salmonicida* by the Mono-As agglutination kit (BIONOR AS) and the respective mutant by streak out on 1% skimmed milk BHI plates and sequencing.

Vaccination experiment

The experimental bacterin based on strain Keldur265-87-6 was prepared from 50 mL broth cultures inoculated with ~10^7 CFU of Keldur265-87-6 toxoid strain and incubated for 96 h resulting in a culture with $OD_{600\ nm}$ = 0.8. To kill bacterial cells, formaldehyde was added to a concentration of 2% (v/v) and cultures were incubated for 24 h at RT. Finally, the culture was dialysed against dH_2O, using a 12-kDa molecular weight cut-off membrane and emulsified with Freund's incomplete adjuvant (FIA) (Sigma-Aldrich, Groco) (1:1), resulting in the experimental bacterin.

Recombinant AsaP1-toxoids, produced as previously described (Schwenteit *et al.* 2013), were emulsified with FIA (1:1), adjusted to a final concentration of 666 μg protein mL^{-1} vaccine. Arctic char (34 ± 7 g) were vaccinated i.p. with 100 μL vaccine/fish (Keldur265-87-6-bacterin, $N = 51$; rec. $AsaP1_{E294A}$ – toxoid, $N = 51$; rec. $AsaP1_{Y309F}$ – toxoid, $N = 50$; PBS + FIA, $N = 51$; PBS, $N = 56$; ALPHAJECT®5-3, $N = 51$). Fish vaccinated with a commercial fish vaccine, ALPHAJECT®5-3 (PHARMAQ), which is a polyvalent bacterin based on *A. salmonicida* subsp. *salmonicida*, *Vibrio anguillarum* O1, *Vibrio salmonicida*, and *Moritella viscosa*, was used as a positive control. PBS and PBS emulsified with FIA served as negative controls. Fish were kept in 400-L tanks with continuously running fresh water at 10 ± 2 °C and a fish density of 3 kg per tank or less. Challenges were performed 12 weeks post-vaccination by i.p. injection of ~10^4 and ~10^5 CFU/fish of the wt strain Keldur265-87. Control fish received PBS only. *A. salmonicida* subsp. *achromogenes* infection was confirmed as described above.

Statistical analysis

Student's *t*-test was used to calculate significances between growth and characteristics of bacterial strains. To compare the MDDs of different groups, Kruskal–Wallis test was applied as oneway ANOVA

Table 3 Percentage accumulated mortality (% mortality), LD_{50}, and mean day of death (MDD) of Arctic char following i.p. injection of toxoid mutant strains Keldur265-87-4 and Keldur265-87-6 and their corresponding complementing mutants Keldur265-87-5 and Keldur265-87-7. The wt strain Keldur265-87 and its isogenic AsaP1-deficient mutant Keldur265-87-2 served as controls, ($n = 10$)

A	Wt Keldur265-87		$AsaP1_{Y309F}$ mutant Keldur265-87-6		$AsaP1_{Y309F}$ complementing mutant Keldur265-87-7	
CFU/fish	% Mortality	MDD	% Mortality	MDD	% Mortality	MDD
~10^6	100	8	100	7		
~10^5	89	12	64	13	78	9
~10^4	67	13	13	10	22	9
~10^3	17	15	17	20	19	12
LD_{50}	1.4 × 10^4 CFU/fish		1.2 × 10^5 CFU/fish		7.3 × 10^4 CFU/fish	
B	AsaP1-deficient mutant Keldur265-87-2		$AsaP1_{E294A}$ mutant Keldur265-87-4		$AsaP1_{E294A}$ complementing mutant Keldur265-87-5	
CFU/fish	% Mortality	MDD	% Mortality	MDD	% Mortality	MDD
~10^6	100	8	100	8	100	7
~10^5	70	9	80	10	90	8
~10^4	60	12	60	11	80	10
~10^3	30	11	30	15	50	11
LD_{50}	1.6 × 10^4 CFU/fish		2.3 × 10^4 CFU/fish		6.4 × 10^3 CFU/fish	

combined with Dunn's multiple comparison test as a post-test to compare all pairs of columns. Fisher's exact test was applied to calculate significances of accumulated mortalities between experimental groups and the Gehan–Breslow–Wilcoxon test was used to compare survival curves. The threshold level for significance was 0.05.

Results

A. salmonicida subsp. *achromogenes* strains producing $AsaP1_{E294A}$- and $AsaP1_{Y309F}$-toxoids instead of the toxic AsaP1 peptidase

Two *A. salmonicida* subsp. *achromogenes* AsaP1-toxoid mutants, Keldur265-87-4 (producing $AsaP1_{E294A}$) and Keldur265-87-6 (producing $AsaP1_{Y309F}$), were successfully constructed and confirmed by sequencing. To assure that the mutant construction caused no other, undesired mutation, the respective complementing mutants, Keldur265-87-5 and Keldur265-87-7, were constructed and analysed in parallel with the toxoid mutant strains.

Both toxoid producing strains, the wt strain, and the complementing mutant had comparable growth rates (Fig. 1). Furthermore, all analysed strains, excluding the AsaP1-deficient strain Keldur265-87-2, secreted the AsaP1-toxin or the corresponding toxoid, detected by α-AsaP1 antibodies in a Western blot (Fig. 2). Toxoid $AsaP1_{Y309F}$ was secreted as 19-kDa peptide similar to the AsaP1 wt (Fig. 2b, lanes 4 and 5), but the toxoid $AsaP1_{E294A}$ was secreted as the unprocessed AsaP1, 37-kDa peptide (Fig. 2a, lanes 3, 4 and 5). Smaller peptides (22 kDa and 19 kDa) were detected in the ECPs obtained from toxoid $AsaP1_{E294A}$ by the specific α-AsaP1 antibody after 168 h of growth (Fig. 2a, lane 5).

Analyses of caseinolytic activity, shown in Fig. 3a, revealed a lack of caseinolytic activity in the ECPs of Keldur265-87-2 (AsaP1-deficient) and Keldur265-87-4 ($AsaP1_{E294A}$–toxoid) and comparable caseinolytic activity of both complementation-mutants as the wt strain Keldur265-87. The azocaseinase assay used was not able to clearly detect caseinolytic activity in the ECP of strain Keldur265-87-6 ($AsaP1_{Y309F}$-toxoid) obtained after 125 h in culture, but a casein zymogram using ECPs obtained after 96-h culture revealed reduced caseinolytic activity in the ECP of strain Keldur265-87-6 (Fig. 3b).

Mortality of Arctic char infected with *A. salmonicida* subsp. *achromogenes* strains producing AsaP1-toxoids

The two toxoid mutant strains, Keldur265-87-4 ($AsaP1_{E294A}$) and Keldur265-87-6 ($AsaP1_{Y309F}$), were used in two separate challenge experiments, A and B, which differed in size of fish

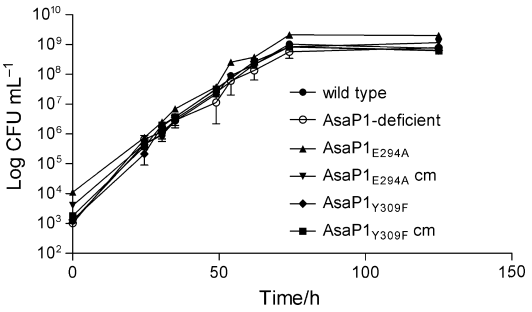

Figure 1 Comparison of growth of different *A. salmonicida* subsp. *achromogenes* strains measured in log10 CFU mL^{-1}, monitored by FACS analyses at different time points over a period of 125 h. The strains are wild type, Keldur265-87; AsaP1-deficient, Keldur265-87-2; AsaP1$_{E294A}$, Keldur265-87-4; AsaP1$_{E294A}$ complementing mutant (cm), Keldur265-87-5; AsaP1$_{Y309F}$, Keldur294-87-6; and AsaP1$_{Y309F}$ cm, Keldur265-87-7.

(A: 44.7 ± 8 g; B: 31.1 ± 7.0 g), water temperature (A: 9 ± 1 °C; B: 11 ± 1 °C) and season (A: February; B: July). Results from comparing the virulence of the wt and mutant strains are shown in Table 3. The LD_{50} calculated 16 day post-infection of strain Keldur265-87-6 (AsaP1$_{Y309F}$-toxoid) in Arctic char (1.2×10^5 CFU/fish) was significantly higher compared with that of the corresponding wt strain, Keldur265-87 (1.4×10^4 CFU/fish) ($P = 0.0302$) (Table 3A). Furthermore, the LD_{50} of strain Keldur265-87-4 (2.3×10^4 CFU/fish) in Arctic char was not significantly different from that of the AsaP1-deficient strain Keldur265-87-2 (1.6×10^4 CFU/fish) ($P = 1.0000$) (Table 3B). This shows that the virulence of the toxoid mutant is comparable with that of the AsaP1-deficient strain. Complementation of the AsaP1 mutation, Keldur265-87-7 ($LD_{50} = 7.3 \times 10^4$ CFU/fish) (Table 3A), was neither significantly different from that of Keldur265-87 nor Keldur265-87-6 ($P = 0.4521$; $P = 0.4571$). Likewise, calculated MDD's evoked by the three analysed strains showed no significant differences ($P = 0.6436$) (Table 3A).

Protection of Arctic char induced by a bacterin based on strain Keldur265-87-6 (AsaP1$_{Y309F}$-toxoid)

The percentage accumulated mortalities of vaccinated fish challenged with the wt strain, Keldur265-87, 12 weeks post-vaccination are shown in Fig. 4. The experimental bacterin based on strain Keldur265-87-6 (AsaP1$_{Y309F}$-toxoid) induced protection in Arctic char ($P < 0.0001$) that was comparable ($P = 0.5676$) with the protection induced by the commercial polyvalent vaccine ALPHAJECT®5–3. The protection raised by the experimental bacterin was significantly better, than that induced by the recombinant AsaP1$_{E294A}$ ($P = 0.0074$) and AsaP1$_{Y309F}$ ($P = 0.0043$) toxoid protein vaccines, respectively. Both AsaP1$_{Y309F}$ and AsaP1$_{E294A}$ toxoid protein vaccines induced significant protection when compared to the PBS control group ($P = 0.0001$ and $P = 0.0002$), but the protection did not differ significantly from that of the PBS/FIA control group ($P = 0.0701$ and $P = 0.0765$), indicating the role of the oil adjuvant in the protection that was induced.

Discussion

In this study, two strains of *A. salmonicida* subsp. *achromogenes*, Keldur265-87-4 and Keldur265-87-6

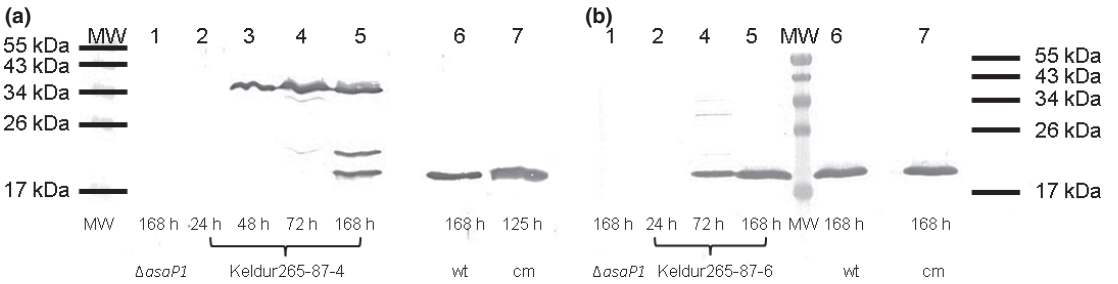

Figure 2 Analyses of AsaP1$_{E294A}$-toxoid (a) and AsaP1$_{Y309F}$-toxoid (b) in B-ECP of *A. salmonicida* subsp. *achromogenes* strain Keldur265-87-4 and Keldur265-87-6, respectively, at several time points (lanes 2-5), using polyclonal murine α-AsaP1 antibodies. B-ECP of the AsaP1-deficient strain Keldur265-87-2 (Δ*asaP1*) served as negative control (a and b lane 1), whereas B-ECP of the wt Keldur265-87 (a and b lane 6) and the respective complementing mutants (cm), Keldur265-87-5 (a lane 7) as well as Keldur265-87-7 (b lane 7) served as positive controls.

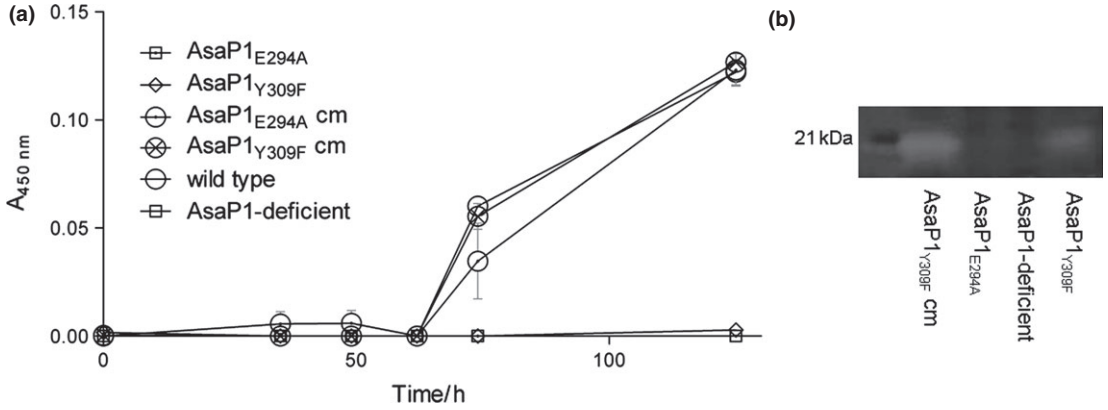

Figure 3 Caseinase activity in ECP of *A. salmonicida* subsp. *achromogenes* AsaP1-toxoid mutant strains (Keldur265-87-4 and Keldur265-87-6), the corresponding complementing mutants (cm) (Keldur265-87-5 and Keldur265-87-7), and as controls wt strain Keldur265-87 and the AsaP1-deficient strain (Keldur265-87-2) measured spectrophotometrically at 450 nm (a); and in a casein zymogram containing 0.1% casein (b).

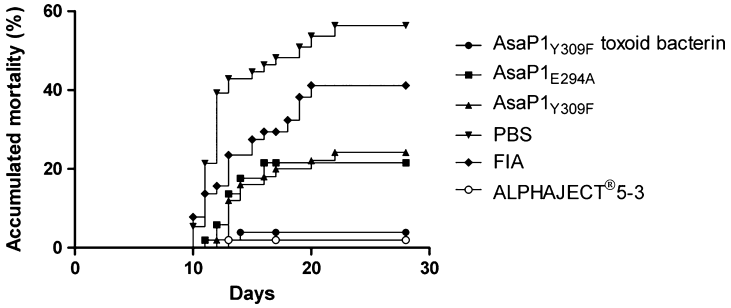

Figure 4 Accumulated mortality of Arctic char challenged by i.p. injection with *A. salmonicida* subsp. *achromogenes* strain Keldur265-87, twelve weeks post-vaccination with experimental vaccines emulsified in an oil adjuvant (FIA). AsaP1$_{Y309F}$-toxoid bacterin, a bacterin based on the toxoid strain Keldur265-87-6 ($N = 51$; $p_{PBS} < 0.0001$, $p_{FIA} < 0.0001$); AsaP1$_{E294A}$ ($N = 51$; $p_{PBS} = 0.0002$, $p_{FIA} = 0.0756$) and AsaP1$_{Y309F}$ ($N = 50$; $p_{PBS} = 0.0001$, $p_{FIA} = 0.0701$) recombinant toxoids 66 μg per fish; and the polyvalent commercial injection vaccine ALPHAJECT®5-3 serving as positive control ($N = 51$; $p_{PBS} < 0.0001$, $p_{FIA} < 0.0001$). Fish injected with PBS ($N = 56$; $p_{FIA} = 0.0574$) and PBS emulsified with FIA ($N = 51$; $p_{PBS} = 0.0574$), served as negative controls.

that secret the toxoids AsaP1$_{E294A}$ and AsaP1$_{Y309F}$, respectively, instead of AsaP1-wt were successfully constructed by allelic exchange. The bacterin based on Keldur265-87-6 was found to protect Arctic char significantly better against atypical furunculosis compared with negative control groups. The protection was comparable with that induced by a commercial polyvalent furunculosis vaccine.

The results of the present study clearly show that AsaP1 is an auto-catalytically processed metalloendopeptidase. The mutant Keldur265-87-4 secretes AsaP1$_{E294A}$-toxoid as a 37 kDa pre-propeptide, which remains unprocessed up to 72 h of cultivation, whereas the still active toxoid AsaP1$_{Y309F}$ is found extracellular as mature 19-kDa enzyme like that of the wt AsaP1 peptidase (Arnadottir *et al.* 2009). In a previous study, recombinant AsaP1$_{E294A}$-toxoid produced by *E. coli* was also expressed as a 37 kDa pre-pro-protein lacking caseinolytic activity and the recombinant AsaP1$_{Y309F}$-toxoid had reduced caseinolytic activity, compared with the recombinant wt protein, but was processed to the mature size of 22 kDa in *E. coli* (Schwenteit *et al.* 2013).

Due to limited wet laboratory conditions, separate mortality experiments were performed for each AsaP1-toxoid strain. Experiment A was performed in February and the tank water was in average 2 °C colder than in experiment B that took place in July. The different physical and

environmental conditions of the fish in the two experiments are unfavourable, and the two experiments cannot be directly compared. The results, however, clearly show that the $AsaP1_{Y309F}$- toxoid mutant has impaired virulence compared with the wt strain, which was recovered in the respective complementing mutant. The AsaP1-deficient strain has been previously shown to have impaired virulence (Arnadottir *et al.* 2009), and in this study, the virulence of the $AsaP1_{E294A}$-toxoid mutant was comparable, but impaired compared with the respective complementing mutant.

Protection of fish immunized with toxoid proteins $AsaP1_{E294A}$ and $AsaP1_{Y309F}$ produced by *E. coli* was comparable, but not significantly better than that induced by the adjuvant alone ($P = 0.701$ and $P = 0.0775$). In our previous study, the $AsaP1_{E294A}$-toxoid was, however, found to induce significantly higher antibody titres in Arctic char than the $AsaP1_{Y309F}$-toxoid (Schwenteit *et al.* 2013). This indicates that the protection is more dependent on T-cell responsiveness than specific antibodies. This is in accordance with studies showing that a live furunculosis vaccine that gave good protection stimulated enhanced T-cell responsiveness in rainbow trout, *Oncorhynchus mykiss* Walbaum (Marsden *et al.* 1996).

Strain Keldur265-87-6 was selected as the component of an experimental bacterin. The selection was based on the results of the virulence studies, the finding that the $AsaP1_{Y309F}$-toxoid is secreted in the size of the mature AsaP1 toxin (19 kDa) and that the two toxoids produced recombinantly in *E. coli* are equivalent in inducing protection in char (our previous unpublished studies retained in this study). The monovalent AsaP1-toxoid bacterin induced a significantly better protection during infection with wt *A. salmonicida* subsp. *achromogenes* compared with negative control groups and the protection provided was comparable with that raised by the commercial polyvalent vaccine. The results are notable, as polyvalent fish vaccines are more efficient in inducing protection compared with monovalent ones (Gudding *et al.* 1997; Hoel, Reitan & Lillehaug 1998).

Toxoids are successfully used to induce protection against well-known human diseases like diphtheria and tetanus (Kitchin 2011). Further, it has been shown that protection induced by the ECP of *A. salmonicida* subsp. *achromogenes* is directly correlated with a raised specific antibody titre against AsaP1 (Gudmundsdottir *et al.* 1997). A vaccine based on an *A. salmonicida* subsp. *achromogenes* strain was commercially available more than a decade ago. It was a monovalent autogenous injection vaccine, IB00 (Alpharma, N. W. Inc.), containing a mineral oil adjuvant. It was found to have relatively poor efficacies in Atlantic salmon, *Salmo salar* L., against a homologous challenge, in comparison with experimental monovalent vaccines produced by our group that had high AsaP1 concentration (Gudmundsdottir *et al.* 1997). Cross protective effects against *A. salmonicida* subsp. *achromogenes* infection in salmon have been reported for the commercial monovalent furunculosis vaccine Bioject 1500, but it induced less protection than the autogenous IB00 vaccine (Gudmundsdottir & Gudmundsdottir 1997). Experimental vaccinations with bacterins based on wt *A. salmonicida* subsp. *achromogenes* have been reported for Atlantic salmon, halibut, *Hippoglossus hippoglossus* L. and cod, *Gadus morhua* L. (Gudmundsdottir & Bjornsdottir 2007; Lund *et al.* 2008a; Lund, Mikkelsen & Schroder 2008b), but there are no previous publications reporting survival of vaccinated Arctic char challenged with *A. salmonicida*. The approach to generate a toxoid-mutant-based bacterin as is presented here is, however, completely new for a furunculosis vaccine. Further, the present study provides new data showing that the polyvalent vaccine ALPHAJECT®5-3 protects Arctic char well against *A. salmonicida* subsp. *achromogenes* infection under the experimental conditions. Survival of char that was vaccinated with the polyvalent ALPHAJECT®5-3 vaccine was 96% and that of the fish vaccinated with the monovalent toxoid-mutant-based bacterin was 92% in a challenge performed 12 weeks from vaccination, and the difference is not significantly different ($P = 0.5676$). As polyvalent fish vaccines are considered to be more efficient than monovalent ones (Gudding *et al.* 1997), it may be expected that vaccination efficacy could be improved if the toxoid-mutant-based bacterin was in mixture with bacterins based on other bacteria in a polyvalent vaccine, but this has to be tested. Challenges of vaccinated fish under experimental and in aquaculture conditions cannot be directly compared. Experimental vaccination experiments, as presented in this study, aim to challenge the fish when the antibody titre is highest. It is also well known that environmental conditions affect the outcome of fish vaccination and that vaccine efficacy declines with time.

In the present study, the bacterin based on an AsaP1-toxoid strain was found to induce good protection in Arctic char and it is highly interesting to analyse its efficacy in inducing protection in other species, especially non-salmonid fish. Further, it would be highly interesting, to evaluate whether the addition of the toxoid mutant in polyvalent furunculosis vaccines would improve their efficacy to protect fish against *A. salmonicida* subsp. *achromogenes* infection.

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